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Endothelin-1 in the regulation of vascular function and glucose metabolism in insulin resistance

by

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To my family

CONTENTS

ABSTRACT

Insulin resistance plays a major role in the pathogenesis of type 2 diabetes and is an important risk factor for cardiovascular disease. Endothelial dysfunction, characterized by reduced bioactivity of nitric oxide and increased activity of the vasoconstrictor and pro-inflammatory peptide endothelin-1 (ET-1), is present in insulin-resistant states and is an important factor promoting the development of cardiovascular complications in patients with insulin resistance. The aim of the thesis was to explore the mechanisms linking insulin resistance to endothelial dysfunction. The hypothesis was that ET-1 via activation of its receptors, ET_A and ET_B, contributes to endothelial dysfunction and reduced insulin sensitivity in subjects with type 2 diabetes mellitus and insulin resistance.

Study I

The effect of the blockade of ET receptors on endothelium-dependent vasodilatation was studied in 12 individuals with insulin resistance without any history of diabetes or cardiovascular disease. Local intra-arterial dual ET_A/ET_B receptor blockade, but not selective ET_A blockade, enhanced forearm endothelium-dependent vasodilatation.

Study II

The importance of endogenous ET-1 for the regulation of total body glucose uptake and insulin sensitivity was studied in 7 patients with insulin resistance and coronary artery disease. Intravenous dual ET_A/ET_B receptor blockade, but not selective ET_A blockade, enhanced insulin sensitivity in this patient group.

Study III

We studied if ET-1 regulates skeletal muscle glucose uptake in 11 insulin resistant subjects *in vivo* and in cultured human skeletal muscle cells. Intra-arterial dual ET_A/ET_B receptor blockade enhanced basal and insulin-stimulated forearm glucose uptake in insulin resistant subjects. ET-1 directly impaired glucose uptake in skeletal muscle cells via a receptor-dependent mechanism.

Study IV

The effect of exogenous ET-1 on basal forearm glucose uptake was studied in 9 subjects with insulin resistance and in cultured human skeletal muscle cells. Intra-arterial ET-1 infusion not only induced vascular dysfunction, but also acutely impaired forearm glucose uptake in individuals with insulin resistance and in skeletal muscle cells from type 2 diabetic subjects. The mechanism seems to be related to signaling downstream of IRS1 Ser^{636}.

Collectively, the obtained data suggest that ET-1 is of pathophysiological importance for the development of endothelial dysfunction and contributes to glucometabolic perturbations in subjects with insulin resistance. Dual ET_A/ET_B receptor blockade may be a potential therapeutic target in order to improve endothelial function and insulin sensitivity in this patient group.

List of Abbreviations

ACE	Angiotensin converting enzyme
Ach	Acetylcholine
AMPK	5’-adenosine monophosphate-activated protein kinase
ATP	Adenosine-5’-triphosphate
BMI	Body mass index
CG	Cardiogreen
CRP	C-reactive protein
ECM	Extracellular matrix
EDV	Endothelium-dependent vasodilatation
EIDV	Endothelium-independent vasodilatation
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular regulated kinase
ET-1	Endothelin-1
FBF	Forearm blood flow
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT4	Glucose transporter type 4
Grb2	Growth factor receptor-bound protein 2
GSK	Glycogen synthase kinase
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HOMA	Homeostasis model assessment
IRS	Insulin receptor substrate
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteases
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PAH	P-aminohippurate
PDGF	Platelet-derived growth factor
PDK	Pyruvate dehydrogenase kinase
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
RBF	Renal blood flow
ROS	Reactive oxygen species
RVR	Renal vascular resistance
SBF	Splanchnic blood flow
Shc	Src-homology-2-containing transforming protein
SNP	Sodium nitroprusside
SOS	Guanine nucleotide exchange factor Son of Sevenless
SplVR	Splanchnic vascular resistance
TG	Triglycerides
TGFβ	Transforming growth factor β
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein

List of Publications

This thesis is based upon the following published papers or manuscripts:

I. **Alexey Shemyakin, Felix Böhm, Henrik Wagner, Suad Efendic, Peter Båvenholm, John Pernow**
Enhanced Endothelium-dependent Vasodilatation by Dual Endothelin Receptor Blockade in Individuals With Insulin Resistance
J Cardiovasc Pharmacol. 2006; 47:385–390

II. **Gunvor Ahlborg, Alexey Shemyakin, Felix Böhm, Adrian Gonon, John Pernow**
Dual endothelin receptor blockade acutely improves insulin sensitivity in obese patients with insulin resistance and coronary artery disease.
Diabetes Care. 2007; 30:591-596.

III. **Alexey Shemyakin, Firoozeh Salehzadeh, Felix Böhm, Lubna Al-Khalili, Adrian Gonon, Henrik Wagner, Suad Efendic, Anna Krook, John Pernow**
Regulation of glucose uptake by endothelin-1 in human skeletal muscle in vivo and in vitro
J Clin Endocrinol Metab. 2010; 95(5):2359-66.

IV. **Alexey Shemyakin, Firoozeh Salehzadeh, Daniella Esteves Duque-Guimaraes, Felix Böhm, Eric Rullman, Thomas Gustafsson, John Pernow, Anna Krook**
Endothelin-1 reduces glucose uptake in human skeletal muscle in vivo and in vitro
Manuscript

INTRODUCTION

General background

Insulin resistance is a pathological condition, which is defined as the reduced responsiveness of insulin-sensitive tissues (mainly liver, skeletal muscle and adipose) or a target cell to insulin exposure. This results in reduced insulin-stimulated glucose uptake in peripheral tissues, the lack of inhibition of hepatic glucose production and, in an effort to maintain glucose homeostasis, a compensatory increase in insulin secretion, resulting in hyperinsulinemia.[1] Insulin resistance plays a major role in the pathogenesis of type 2 diabetes, a metabolic disorder characterized by chronic hyperglycemia, with disturbances of carbohydrate, fat and protein metabolism, resulting from the defects of insulin action.[2] Type 2 diabetes comprises 90% of people with diabetes around the world. It is associated with reduced life expectancy and significant morbidity due to specific diabetes related microvascular complications, increased macrovascular complications (ischemic heart disease, stroke and peripheral vascular disease), and diminished quality of life. Recent estimates indicate there were 246 million people in the world with diabetes in the year 2007[3] and this is expected to increase to at least 366 million by 2030.[4] Insulin resistance is present not only in the majority of patients with type 2 diabetes, but also in the early pre-diabetic state of impaired glucose tolerance and in individuals with normal glucose tolerance who are the offspring of patients with type 2 diabetes.[5] For this reason, insulin resistance not only plays a major role in the pathogenesis of type 2 diabetes, it is also an initial measurable defect predicting the development of type 2 diabetes.[6] Moreover, insulin resistance is an important risk factor for cardiovascular disease,[7-9] which is the major cause of death and disability in patients with type 2 diabetes[10] and the leading cause of overall morbidity and mortality in the developed countries.[11] Insulin resistance is also closely associated with other major public health problems, including obesity, hypertension and dyslipidemia. A global obesity epidemic is currently driving the increased incidence and prevalence of insulin resistance and its cardiovascular complications.[12] There are several reasons why patients with insulin resistance and type 2 diabetes are particularly prone to develop coronary events. They include disturbed platelet function, reduced fibrinolytic capacity, dyslipidemia and hyperglycemia.[13, 14] Interestingly, all these conditions may contribute to a state that is usually referred to as "endothelial dysfunction". This term refers to the dysfunctional endothelium with an impaired ability to maintain vascular homeostasis. Endothelial dysfunction contributes to the pathogenesis of atherosclerosis,[15] insulin resistance[16] and vascular disease in type 2 diabetes.[17] Furthermore, it is closely related to clinical events in patients with atherosclerosis, hypertension and type 2 diabetes.[10, 18] Endothelial dysfunction could therefore be one of the possible mechanisms leading to the development of atherosclerosis and its clinical complications in patients with insulin resistance. The objective of this thesis is to explore the mechanisms linking insulin resistance to endothelial dysfunction.

The endothelium and endothelial function

The endothelium, which is a monolayer of endothelial cells, is localized at the interface between the vessel wall and circulating blood. The discovery almost three decades ago that the endothelium is not an inert semi-permeable barrier that prevents the leakage of excessive

plasma fluid through the monolayer but is also able to elicit vasodilatation[19] led to a revolution in vascular biology. In fact, by producing a large number of biologically active substances, the endothelium plays a pivotal role in cardiovascular homeostasis. It participates in the regulation of vascular tone, thrombosis and hemostasis, vascular permeability, blood pressure, the recruitment and activity of inflammatory cells and cell growth. Endothelial cells are able to produce both vasodilator and vasoconstrictor substances. The main endothelium-derived relaxing factors are nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor. The main vasoconstricting substances produced by the endothelium are thromboxane A2, angiotensin II and endothelin (ET)-1. The release of endothelium-derived vasodilators can be induced by several physiological and pharmacological factors, such as acetylcholine, serotonin, angiotensin II, alpha adrenergic agonists and increased shear stress. When affecting vascular smooth muscle, the very same factors promote vasoconstriction. The vascular endothelium thus plays an important role in maintaining the delicate balance of vascular tone.

The gaseous molecule NO – the most potent endothelium-derived vasorelaxing substance and an important determinant of endothelial function – is generated from the conversion of the amino acid L-arginine by endothelial NO synthase (eNOS). Classical cholinergic vasodilators (e.g. acetylcholine) activate G-coupled receptors on endothelial cells, which results in a rise in intracellular calcium levels. The calcium/calmodulin complex interacts with the calmodulin-binding site on eNOS, thereby increasing its enzymatic activity. Another pathway leading to eNOS activation is the direct phosphorylation of eNOS at Ser^{1177} by different serine kinases, including Akt, 5' adenosine monophosphate-activated protein kinase (AMPK) and protein kinase A (PKA). The availability of L-arginine and enzymatic cofactors (e.g. tetrahydrobiopterin) also plays an important role in the regulation of NO production.[20] Once formed in endothelial cells, NO diffuses freely into adjacent vascular smooth muscle cells, where it activates guanylate cyclase which in turn increases guanosine monophosphate levels to induce vasodilatation. In addition to its vasorelaxing action, NO can also exhibit other vasoprotective properties such as the attenuation of inflammation. This effect is obtained by reducing the expression of different adhesion molecules. They include vascular cell adhesion molecule-1 (VCAM-1), E-selectin and intercellular adhesion molecule-1 (ICAM-1).[21] NO also inhibits the production and/or release of several inflammatory cytokines and chemokines, such as tumor necrosis factor-α, monocyte chemoattractant protein-1,[22] tissue factor,[23] interleukin-6 and interleukin-8.[21] As a result, NO attenuates the binding of inflammatory cells such as monocytes and macrophages to the vascular wall, as well as platelet adhesion.[24] These effects are attributed to the ability of NO to inhibit actions of the transcription factor nuclear factor kappa B.[25]In addition, NO inhibits vascular smooth muscle cell growth and proliferation[26, 27] and intimal hyperplasia, which involves both the proliferation and migration of vascular smooth muscle cells.[28] In the cellular environment, NO may react with reactive oxygen species (ROS) to form strong oxidant intermediates such as peroxynitrite ($ONOO^-$) via the reaction with superoxide. The inactivation of NO by the enhanced production of ROS in the vasculature can significantly reduce NO bioavailability. Under physiological conditions, adequate levels of NO are maintained by the efficiency of antioxidant enzymes that quench ROS production and thereby limit peroxynitrite formation.[29] The bioavailability of NO, i.e. the concentrations of free NO available to produce a biological response, is therefore dependent on the balance between the production by eNOS and the inactivation of NO by oxidative processes.

In the healthy artery, the balance of vasoactive substances produced by the endothelium favors vasorelaxation and anti-thrombotic and anti-inflammatory effects (Fig 1A). In disease, there is a shift towards the reduced bioavailability of NO, which in turn results in enhanced vascular tone, increased oxidative stress, platelet activation and inflammatory activity (Fig 1B). This maladapted endothelial phenotype, referred to as "endothelial dysfunction", is present in many cardiovascular and metabolic disorders, such as hypertension,[30] coronary artery disease,[15] dyslipidemia,[31] obesity,[32] insulin resistance[33] and type 2 diabetes.[17] Almost 25 years ago, Ludmer *et al.* demonstrated that prestenotic and stenotic segments of coronary arteries exhibited a paradoxical vasoconstriction in response to the intra-arterial infusion of acetylcholine.[15] In fact, endothelial dysfunction has been shown to be one the earliest manifestations of the atherosclerosis process, where abnormal vasoconstriction can be observed at the site of future plaque development.[34] It is important to note that the impairment of endothelial function involves several other biological processes apart from the reduced bioavailability of NO. One of them is the increased production and biological activity of ET-1, a peptide with potent vasoconstrictor and pro-inflammatory properties.[35]

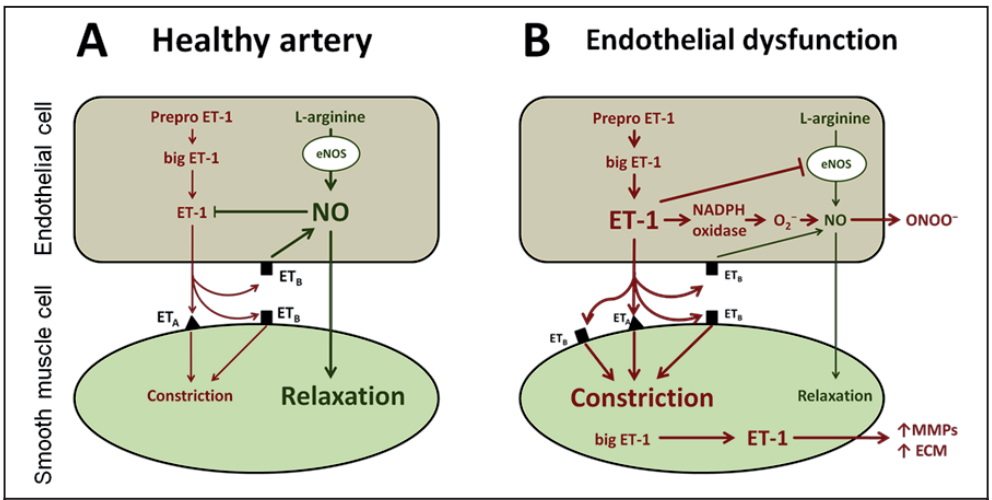

Figure 1. Schematic figure of the arterial wall under healthy conditions (left) and in endothelial dysfunction (right). In healthy arteries, the production of ET-1 is small and the bioavailability of nitric oxide (NO) is preserved, which favors vasorelaxation. In endothelial dysfunction, the expression of ET-1 in smooth muscle cells is increased. The expression of ET_B receptors on smooth muscle cells is increased, leading to increased vasoconstriction. ET-1 may reduce endothelial NO synthase (eNOS) expression, which results in reduced NO production. ET-1 may mediate the formation of superoxide ($O2^-$), thereby reducing the biological activity of NO by forming peroxynitrite ($ONOO^-$). ET-1 stimulates the production of extracellular matrix (ECM) and matrix metalloproteases (MMPs), resulting in vascular remodeling.

Endothelin-1 and its role in endothelial dysfunction

ET-1 belongs to the family of endothelins which comprises three vasoactive peptides, ET-1, ET-2 and ET-3. ET-1, described by Yanagisawa *et al.* in 1988,[36] is regarded as the most important endothelin isoform for the cardiovascular system.[37] ET-1 is synthesized in endothelial cells from its inactive precursors prepro ET-1 and big ET-1, processed by a subgroup of

membrane-bound zinc metalloproteases, the endothelin-converting enzymes (ECEs), into the active ET-1. ET-1 is also produced in other cell types such as vascular smooth muscle cells, cardiac myocytes,[38] macrophages[39] and leukocytes.[40] ET-1 acts mainly in a paracrine manner, exerting its effects via the activation of two G-protein coupled receptors, namely the ET_A and ET_B receptor subtypes, which are located on vascular smooth muscle cells (both ET_A and ET_B) and endothelial cells (ET_B only). The stimulation of both receptor subtypes on vascular smooth muscle cells leads to vasoconstriction via intracellular calcium release, while the stimulation of ET_B receptors on endothelial cells leads to vasodilatation due to the release of NO and prostacyclin (Fig. 1A). ET-1 stimulates vascular smooth muscle cells proliferation,[41] the migration,[42] contraction[43] and expression of pro-atherogenic growth factors like platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)β.[44, 45] In vascular smooth muscle cells and fibroblasts, the expression of extracellular matrix and matrix metalloproteinases is stimulated by ET-1 via the ET_A-mediated pathway, which may lead to the promotion of tissue remodeling.[46, 47]

Many pathological conditions, such as type 2 diabetes,[48] obesity,[49] essential hypertension[50] and coronary artery disease,[51] are associated with elevated plasma levels of ET-1. A complex interaction between ET-1 and NO appears to exist in the vascular wall. Endogenous NO is known to down-regulate ET-1 secretion.[52] On the other hand, ET-1 impairs endothelium-dependent vasodilatation induced by NO.[53] Accordingly, ET receptor blockade improves endothelium-dependent vasodilatation in patients with atherosclerosis[54] and hypertension.[55] There could be several reasons for these effects. First, it has been demonstrated that the dual ET receptor antagonist bosentan increases the expression of eNOS.[56] Furthermore, eNOS activity is stimulated by dual ET receptor blockade.[57] Second, increased ROS production due to the excessive stimulation of both ET_A and ET_B receptors could result in increased NO degradation with the formation of peroxynitrite. Moreover, ET-1 induces the up-regulated expression of caveolin-1 − the major coat protein of caveolae, which appears to be a key negative regulatory protein for eNOS activity[58] − leading to the inhibition of eNOS activity.[59, 60]

It is often assumed that most of the negative effects of ET-1 are mediated via the stimulation of the ET_A receptor. In fact, in physiological conditions, vasoconstriction is mainly mediated by the ET_A receptor, which is partly counteracted by the release of NO mediated by ET_B receptors located on endothelial cells. Selective ET_A receptor antagonism has been reported to have positive effects on vascular function. The ET_A receptor antagonist BQ123 evokes an increase in forearm blood flow in healthy men.[61] Conversely, in another report, BQ123 increased forearm blood flow only in hypertensive patients but not in normotensive controls.[62] Furthermore, hypertensive patients with obesity respond to ET_A receptor antagonism with a higher increase in blood flow, compared with lean hypertensive patients.[63] Selective ET_A receptor blockade was reported to improve nutritive skin capillary circulation in patients with type 2 diabetes and microangiopathy.[64] At the same time, ET_B receptor antagonism (alone or in combination with ET_A receptor antagonism) was shown to induce local vasoconstriction in young healthy subjects.[65] Possible explanations for this effect are that the ET_B receptor antagonist blocks the vasodilator ET_B receptor on endothelial cells and that ET_B receptors are of importance for the clearance of ET-1.[66] It has therefore been speculated that the blockade of endothelial ET_B receptors may be detrimental for vascular function.[57] On the other hand, there are indications of an up-regulation of vasoconstricting ET_B receptors on vascular smooth muscle cells that may contribute to vascular dysfunction in pathological

conditions. There is evidence of the increased expression of ET_B receptors in atherosclerotic human arteries,[67] as well as in a mouse model overexpressing ET-1.[68] Accordingly, dual ET_A/ET_B receptor blockade increases endothelial NO synthase activity more than selective ET_A receptor blockade in hypercholesterolemic pigs[69] and dual ET_A/ET_B receptor blockade results in more pronounced vasodilatation than selective ET_A receptor blockade in patients with atherosclerosis.[70] It can therefore be speculated that dual ET_A/ET_B receptor antagonism is beneficial in vascular dysfunction associated with atherosclerosis. The effect of dual ET_A/ET_B receptor blockade on vascular function in insulin resistance and type 2 diabetes remains to be clarified.

Insulin actions and signaling in vascular endothelium in health and disease

Insulin is a powerful anabolic hormone whose primary role is to promote fuel storage (by increasing glycogen synthesis in the liver and muscle, augmenting triglyceride synthesis and deposition in adipose tissue and promoting protein synthesis and inhibiting proteolysis) and enhance glucose oxidation, providing an important energy source in the form of adenosine-5'-triphosphate (ATP). To exert these actions, insulin needs to cross the endothelial barrier to reach its target receptor in insulin-sensitive tissues. The exact mechanism has not yet been fully elucidated, but the insulin molecule is thought to be internalized by vascular endothelial cells via a receptor-mediated process.[71] Insulin receptors are expressed at the cell surface plasma membrane. The insulin receptor consists of four subunits, two extracellular insulin-binding α-peptides linked with two transmembrane β-peptides. On the intracellular side of the β-subunits, there is a tyrosine kinase domain. When insulin binds to the receptor, conformational change results in the activation of the β-subunits through autophosphorylation on tyrosine residues, providing docking sites for the binding of down-stream signaling molecules. There are two major branches of the complex insulin signal transduction network.

I) The metabolic branch stimulates glucose transporter type 4 **(**GLUT4) translocation to the cell membrane and thereby regulates glucose uptake[72] in adipocytes and myocytes. This action is mediated by the activation of the insulin receptor substrate (IRS)-1, which results in the downstream activation of phosphatidylinositol 3-kinase (PI3-K) and the subsequent phosphorylation of the serine/threonine kinase Akt.[73, 74] The effect of Akt on glucose uptake has been linked to the phosphorylation of its substrate AS160[75] (also called TBC1D4), which regulates Rab proteins involved in GLUT4 vesicle translocation.[76] Furthermore, Akt phosphorylates glycogen synthase kinase-3 (GSK-3) resulting in glycogen synthesis.[77] Additional Akt-independent pathways have been implicated in the mediation of insulin-stimulated glucose transport, involving the phosphoinositide-dependent protein kinase-1 (PDK)-1 and protein kinase C (PKC) isoforms ζ/λ (Fig. 2).[78]

II) The mitogen-activated protein kinase (MAPK)-dependent pathway is involved in insulin signaling related to gene expression, mitogenesis, cell growth and differentiation.[79] Interestingly, this pathway is also implicated in the insulin-stimulated secretion of ET-1 (Fig. 2).[80]

The insulin-mediated stimulation of the PI3-K pathway in the vascular endothelium, which exhibits striking parallels with the metabolic insulin signaling pathway in muscle and adipose tissue, results in the phosphorylation of serine-threonine kinases including Akt and the direct phosphorylation of eNOS (Fig. 2).[81, 82] This leads to the stimulation of NO production,

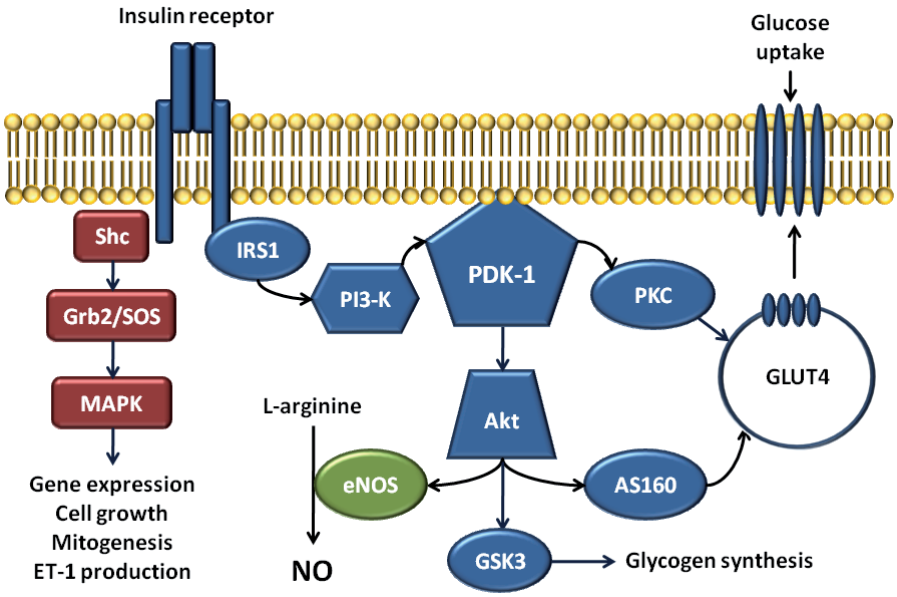

Figure 2. Schematic view of the insulin signaling pathway. The phosphatidylinositol 3-kinase (PI3-K) branch regulates glucose transporter type 4 **(**GLUT4) translocation and nitric oxide (NO) production via phosphorylation of the endothelial nitric oxide synthase (eNOS). It also regulates glycogen synthesis via phosphorylation of the glycogen synthase kinase 3 *(*GSK3). The mitogen-activated protein kinase (MAPK) branch, which include Src-homology-2-containing transforming protein (Shc), growth factor receptor-bound protein 2 (Grb2) and guanine nucleotide exchange factor (SOS), regulates gene expression, cell growth, mitogenesis and the production of endothelin-1 (ET-1).

vasodilatation, increased capillary recruitment and elevated total blood flow.[83] These vascular actions augment the delivery of insulin and glucose to skeletal muscle, thereby enhancing glucose uptake and utilization. It has been proposed that as much as 25% of the stimulatory effect of insulin on muscle glucose uptake is related to its hemodynamic actions.[84] The specificity of this NO-dependent mechanism has been confirmed using a specific inhibitor of eNOS (N^G-monomethyl-L-arginine) which substantially reduced insulin-mediated vasodilatation.[85] The same effect could be obtained following the inhibition of the essential co-factor of NO synthesis, tetrahydrobiopterin.[86] At the same time, insulin-stimulated NO production could be antagonized by inhibitors of PI3-kinase or Akt.[87, 88] In addition to this vasodilating effect, insulin also exerts vasoconstricting effects by stimulating the sympathetic nervous system[89] and the release of ET-1.

The interactions between insulin and vascular function are complex and may differ between healthy and insulin-resistant states. In obese subjects with insulin resistance, as compared to insulin-sensitive lean subjects, impaired insulin diffusion across the vascular endothelium was described, representing a potential rate-limiting step in peripheral insulin action.[90] The vasodilatory effect of insulin is blunted due to alterations in insulin signaling in obese

subjects and patients with type 2 diabetes,[91] as well as in animal models of insulin resistance.[92] Furthermore, in insulin-resistant states of obesity and type 2 diabetes, an impaired response to different endothelium-dependent vasodilators (acetylcholine, metacholine and bradykinin) has also been reported.[93] On a molecular level, metabolic insulin resistance results from impaired PI3-K-dependent signaling − the same pathway that is involved in the regulation of NO production in the endothelium − thereby leading to the reduced bioavailability of NO. Coexisting hyperinsulinemia results in the activation of genes involved in inflammation[94] and excessively activates the MAPK pathway in a pro-atherogenic manner: IRS1 interacts with Src-homology-2-containing protein (Shc), leading to the activation of extracellular regulated kinase (ERK), which catalyses the phosphorylation of transcriptional factors that promote cell growth, cell proliferation, cell differentiation and ET-1 production (Fig. 2).[81, 82, 95]

Other factors aggravating endothelial dysfunction in insulin resistance

Insulin resistance most often precedes impaired glucose tolerance and hyperglycemia.[96] So, despite the fact that hyperglycemia plays a major role in the development of endothelial dysfunction in type 2 diabetes via the activation of different signaling pathways leading to increased oxidative stress,[13] it is unlikely that this is the case at the pre-diabetic stage. Abdominal obesity accompanied by dyslipidemia and low-grade inflammation is a common feature in insulin resistance, which could also promote the development of endothelial dysfunction. Increased levels of circulating free fatty acids and triglycerides induce a reduction in vascular reactivity, presumably via both endothelium-dependent and endothelium-independent mechanisms.[97] In insulin-resistant subjects, serum levels of free fatty acids are generally increased, resulting in the excessive production of superoxide and the reduction of eNOS activity.[98] The increased generation of ROS could additionally aggravate endothelial function in insulin-resistant states due to the reduction of NO bioavailability.[99] In recent studies, high-density lipoprotein (HDL) cholesterol and adiponectin were shown to stimulate the production of NO from vascular endothelium by a PI3-K-dependent mechanism.[100, 101] Reduced levels of HDL cholesterol and adiponectin may therefore contribute to the decrease in NO bioavailability. Additionally, increases in the levels of very-low-density lipoprotein (VLDL) particles are correlated to endothelial dysfunction.[102] Many studies have demonstrated that insulin by itself is able to promote atherogenesis via several mechanisms, such as the stimulation of de novo lipogenesis, the enhancement of VLDL synthesis, increased cholesterol transport in arteriolar smooth muscle cells, the augmentation of collagen synthesis and the stimulation of the proliferation of arteriolar smooth muscle cells.[103] The production of proinflammatory cytokines, such as tumor necrosis factor α (TNFα), is increased in obesity. TNFα may selectively inhibit the PI3-kinase signaling pathway and induce endothelial dysfunction by altering the balance between NO and ET-1.[104]

ET-1 and the regulation of glucose uptake

Recent studies suggest that ET-1 may be involved in the regulation of glucose uptake by directly inhibiting insulin-mediated glucose uptake via a receptor-dependent mechanism. Cell culture studies demonstrate that ET-1 impairs insulin-stimulated glucose transporter GLUT4 translocation in adipocytes[105, 106] and reduces PI3-kinase activity via IRS2 serine and tyrosine phosphorylation in isolated vascular smooth muscle cells.[107] There is also evidence that ET-1 impairs GLUT4 trafficking via PI3-kinase independent membrane-based signal transduction Cbl associated protein (CAP)/Cbl pathway.[106] In isolated rat soleus muscle strips, ET-1 pretreatment for one hour leads to a reduction in insulin-stimulated glucose transport. In

the same animal model, chronic ET-1 administration in vivo leads to whole-body insulin resistance, with decreased skeletal muscle glucose transport and impaired insulin signaling (reduction in the expression of IRS1 protein and IRS1-associated p110alpha phosphoinositide 3-kinase, as well as Akt activation).[108] In humans, Ferri *et al.*[48] demonstrated a negative correlation between total glucose uptake and circulating ET-1 levels in type 2 diabetes. Furthermore, ET-1 interferes with glucose metabolism as shown by a drop in splanchnic glucose production and peripheral glucose utilization during ET-1 infusion in healthy subjects.[109] The notion that ET-1 modulates insulin sensitivity was supported by the finding that ET-1 reduced insulin sensitivity in healthy volunteers.[110] Furthermore, the ET-1 precursor, big ET-1, reduced insulin sensitivity via an action mediated by the ET_A receptor in healthy subjects.[111] Selective ET_A receptor blockade was shown to augment insulin-mediated glucose uptake in obese but not lean subjects.[112] The metabolic role of ET-1 in relation to its vascular effects in subjects with insulin resistance is mainly unknown, however. Further studies are therefore needed to elucidate the involvement of ET-1 in the regulation of glucose uptake in subjects with glucometabolic perturbations.

AIMS

The overall aim of these studies was to test the hypothesis that ET-1 is of importance for the development of endothelial dysfunction and insulin resistance. Based on this, the specific aims were to investigate:

1. Whether ET receptor blockade enhances endothelium-dependent vasodilatation in individuals with insulin resistance and whether such an effect is mediated via selective ET_A or dual ET_A/ET_B receptor blockade **(I)**
2. Whether ET (selective ET_A and dual ET_A/ET_B) receptor blockade improves insulin sensitivity in patients with insulin resistance and coronary artery disease **(II)**
3. Whether ET-1 regulates skeletal muscle glucose uptake *in vivo* and *in vitro* **(III, IV)**
4. The molecular mechanisms behind the effect of ET-1 on glucose uptake in cultured skeletal muscle cells **(III, IV)**

MATERIAL AND METHODS

Study subjects

All the investigations were carried out in accordance with the Declaration of Helsinki and were approved by the ethics committee at Karolinska Institutet. The participants were informed of the nature, purpose and possible risk involved in the study before giving informed consent.

Study I

Twenty male subjects were recruited from the registry at the Department of Endocrinology at Karolinska University Hospital in Stockholm. All the subjects were clinically healthy and had no previous history of any cardiovascular disease. All the participants had previously undergone an evaluation of insulin sensitivity using the hyperinsulinemic-euglycemic clamp method. Subjects with an M-value of <6 or >11 underwent a second clamp within the protocol of this study to create two separate groups with an insulin-resistant and an insulin-sensitive phenotype. Baseline characteristics of the subjects are presented in Table 1. Twelve subjects with a low M-value (insulin resistant) and eight subjects with a high M-value (insulin sensitive) were included. Individuals with insulin resistance had significantly higher plasma glucose than the insulin-sensitive individuals at 120 min following an oral glucose loading (OGTT). Furthermore, the insulin-resistant group had higher insulin levels at baseline and after 30 and 120 min following the oral glucose loading than the insulin-sensitive group. Body mass index, total cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides (TG) were significantly higher in the insulin-resistant group (Table 1). There were no significant differences in HDL levels between the two groups (Table 1).

Study II

Seven patients (58±2 yr, BMI 31.7±2.6) with a previous history of impaired glucose tolerance and coronary artery disease were recruited. Impaired glucose tolerance was defined as blood glucose of ≥7.8 mmol/l two hours after an oral glucose loading (75 g). Patients were classified as having diabetes mellitus if their fasting blood glucose exceeded 6.0 mmol/l (on at least two occasions) or their blood glucose concentration was >11.0 mmol/l two hours after an oral glucose loading. Based on these criteria, five patients were classified as diabetic and two as having impaired glucose tolerance. Coronary artery disease was defined as a history of previous myocardial infarction or significant coronary stenosis verified by coronary angiography. The patients were taking aspirin (n=7), statins (n=6), fibrates (n=1), angiotensin-converting enzyme (ACE) inhibitors (n=5), beta-blockers (n=7) and oral anti-diabetics (n=5). Their average total, LDL and HDL cholesterol levels were 4.0±0.3, 2.3±0.3 and 0.9±0.1 mmol/l respectively. Their average serum creatinine was 87±5 μmol/l.

Study III

Eleven sedentary male subjects (61±3 yr, BMI 28.4±1.6 kg/m^2) with insulin resistance as assessed by either hyperinsulinemic-euglycemic clamp (total body glucose uptake <6 mg/kg/min; n=8) or homeostasis model assessment of insulin resistance (HOMA >2.5; n=3) were recruited. Six of the subjects had hypertension and two had had a prior myocardial infarction. The subjects were taking aspirin (n=1), ACE inhibitors or angiotensin receptor antagonists

Table 1. Baseline characteristics of subjects in Study I.

	Insulin resistant (n=12)	Insulin sensitive (n=8)	*p*-value
M-value (mg/kg/min)	5.5 ± 1.9	12.5 ± 3.9	<0.001
Age (years)	53 ± 6	54 ± 4.7	=ns
Weight (kg)	90 ± 10	82 ± 14.6	=ns
Height (m)	1.80 ± 0.1	1.85 ± 0.08	=ns
Body mass index (weight/height^2)	27.8 ± 3.4	23.8 ± 3.0	<0.05
Mean arterial pressure (mmHg)	93 ± 9	93 ± 9	=ns
Smokers (no.)	0	0	
Ex-smokers (no.)	6	3	
Non-smokers (no.)	6	5	
Total cholesterol (mmol/l)	6.4 ± 0.5	5.5 ± 1.0	<0.01
LDL (mmol/l)	4.5 ± 0.5	3.6 ± 0.8	<0.01
HDL (mmol/l)	1.2 ± 0.3	1.4 ± 0.3	=ns
TG (mmol/l)	1.6 ± 0.5	0.9 ± 0.2	<0.01
OGTT 0' (plasma glucose, mmol/l)	5.2 ± 0.8	5.2 ± 0.4	=ns
OGTT 30' (plasma glucose, mmol/l)	8.2 ± 1.5	7.9 ± 0.7	=ns
OGTT 120' (plasma glucose, mmol/l)	7.5 ± 1.4	5.5 ± 0.9	<0.01
Insulin 0' (μU/ml)	15.2 ± 5.9	8.9 ± 2.4	<0.01
Insulin 30' (μU/ml)	66.7 ± 38.0	35.8 ± 9.4	<0.05
Insulin 120' (μU/ml)	94.0 ± 71.0	37.2 ± 13.9	<0.01

Values are means ± SD

(n=2), beta-blockers (n=1) or calcium channel blockers (n=1). Their average total LDL and HDL cholesterol levels were 5.1±0.4, 3.2±0.2 and 1.2±0.4 mmol/l respectively. Their average triglyceride level was 1.4±0.6 mmol/l. Their average glycated hemoglobin (HbA1c), C-reactive protein (CRP) and plasma ET-1 levels were 4.7±0.2 %, 1.8±1.3 mg/l and 2.9±0.3 pmol/l respectively. For the *in vitro* part of the study, muscle biopsies (*rectus abdominis*) were obtained from 8 subjects during planned abdominal surgery. The subjects did not have any metabolic disorders.

Study IV

Nine sedentary male subjects (62±7 yr, BMI 27.8±1.9 kg/m^2) with insulin resistance as determined by either euglycemic-hyperinsulinemic clamp (total body glucose uptake <6 mg/kg/min; n=6) or homeostasis model assessment of insulin resistance (HOMA >2.5; n=3), who participated in Study III, were recruited for this study. For the *in vitro* part of the study, muscle biopsies (*rectus abdominis*) were obtained from 19 subjects without any metabolic disorders during planned abdominal surgery (58±3 yr, BMI <25 kg/m^2). To investigate differences between subjects with type 2 diabetes and healthy controls, muscle biopsies (*vastus lateralis*)

were also obtained from 11 male subjects with type 2 diabetes (60±2 yr, BMI 29±1 kg/m^2, HOMA=4.3±1.9) and 11 male subjects with normal glucose tolerance (64±1 yr, BMI 28±1 kg/m^2, HOMA=2.0±0.5) under local anesthesia (Lidocaine hydrochloride 5 mg/ml) following an overnight fast.

Blood flow measurements

All the investigations were performed with the subjects in the supine position in a quiet laboratory with a controlled temperature. The subjects arrived at the laboratory in the morning, were instructed not to use caffeine and nicotine-containing products on the study day and all medication was withheld on the study day.

Venous occlusion plethysmography (I, III, IV)

Forearm venous occlusion plethysmography is one of the "gold standards" in the assessment of vascular function and is an accurate, reproducible method with which to assess the effect of vasoactive substances in humans *in vivo*. This method involves tying a strain gauge – a stretchable tube containing mercury – around the limb. When venous drainage from the arm is briefly interrupted with inflatable cuffs, arterial inflow remains unaffected, thereby resulting in a linear increase in forearm volume over time, which is proportional to arterial blood inflow, until venous pressure rises towards the occluding pressure. Changes in limb circumference alter the cross-sectional area of the strain gauge and thereby the electrical resistance of the mercury, which can be recorded as an analog voltage signal. In our studies, forearm blood flow (FBF) was measured simultaneously in both arms using a mercury-in-silastic strain gauge applied around the widest part of the forearm. A cuff placed around the upper arm was inflated to 50 mmHg for 10 sec to obstruct the venous outflow during the recording of FBF. The circulation of the hands was occluded by inflating a wrist cuff to 30 mmHg above systolic blood pressure. FBF values were obtained from four to eight inflow measurements during two minutes of recording. A percutaneous catheter was inserted under local anesthesia in the proximal direction into the brachial artery of the non-dominant arm for infusions and blood sampling. Another catheter was inserted in the distal direction of a deep cubital vein, draining mainly skeletal muscle tissue, on the ipsilateral arm for collection of blood samples. Endothelium-dependent vasodilatation (EDV) was determined by an infusion of acetylcholine into the brachial artery. This was followed by an infusion of the NO donor sodium nitroprusside (SNP) for the determination of endothelium-independent vasodilatation (EIDV).

Dye dilution technique (II)

The dye dilution method for measuring blood flow is based on rapidly injecting a known quantity of a dye at one site into the circulatory system and withdrawing blood at a distal site to determine a concentration curve for the dye. Two thin catheters were inserted percutaneously into one antecubital vein on each arm for infusions. Another catheter was introduced into the brachial artery for sampling blood and measuring systemic arterial blood pressure. Splanchnic (SBF) and renal blood flows (RBF) were determined by the constant infusion of cardiogreen (CG), and *p*-aminohippurate (PAH) and the hematocrit. Sixty minutes after catheterization (and the initiation of the PAH and, 10 min later, the CG infusion), blood samples for the determination of CG and PAH were drawn from the catheters for basal measurements.

This was followed by sampling every 20 min up to 60 min. This method has been evaluated previously using the introduction of hepatic and renal vein catheters to ascertain that fractional uptake, equal to the arterio-venous difference divided by the arterial concentration, of CG and PAH was not influenced by the infusion of the ET-1 blockers[113] or the clamp procedure.[111] The hematocrit was analyzed with a microcapillary hematocrit centrifuge and corrected for trapped plasma. Splanchnic (SplVR) and renal vascular resistances (RVR) were calculated as mean arterial pressure divided by SBF or RBF respectively and measured at the baseline and thereafter every 20 min.

Glucose uptake measurements

Hyperinsulinemic-euglycemic clamp (II)

The hyperinsulinemic-euglycemic clamp is the "gold standard" for investigating and quantifying total body glucose uptake and insulin sensitivity. The principle of this method is to measure the amount of glucose necessary to compensate for an increased insulin level without causing hypoglycemia. Two thin catheters were inserted percutaneously into one antecubital vein on each arm for infusions. Another catheter was introduced into the brachial artery for blood sampling. Insulin, dissolved in 0.9% saline and blood, was infused at a rate corresponding to 804 mU/m^2 body surface area during the first 8 min, followed by 40 mU/m^2/min for 112 min. The fasting blood glucose level was maintained by adjusting the infusion rate of a 20% glucose solution. Arterial blood samples were taken every 5 min for the determination of blood glucose.

Forearm glucose uptake (III, IV)

Since skeletal muscle accounts for a major part of glucose uptake, the rate of glucose utilization in skeletal muscle is of great importance for the determination of insulin sensitivity. We therefore used the forearm model in which it is possible to determine local arterio-venous concentration differences and blood flow in order to calculate glucose uptake in a vascular bed mainly supplying skeletal muscle.[114] During venous occlusion plethysmography, FBF was measured and arterial and deep venous blood samples were collected. Forearm glucose uptake (FGU) was calculated according to the following formula: (arterial – venous glucose concentration) × blood flow × (1-hematocrit).

Glucose uptake in skeletal muscle cell cultures (III, IV)

Glucose uptake in skeletal muscle cell cultures was assessed using isotope-labeled glucose. Muscle biopsies were collected in cold PBS supplemented with 1% PeSt (100 U/ml penicillin and 100 μg/ml streptomycin). Satellite cells were isolated and cultured to form myotubes as described.[115] Myotubes were incubated in serum-free medium overnight before each experiment. ET-1 or vehicle was added in the absence or presence of the dual ET_A/ET_B receptor antagonist bosentan (3 μM) for the times indicated. Bosentan was added 30 min before ET-1. Control cells were exposed to the vehicle for the same length of time. Where indicated, insulin (60 nM) was added for 30 min. Overnight serum-starved myotubes (in the presence or absence of ET-1) were stimulated with or without insulin in KREBS buffer. Cells were then incubated with 10 μM 2-deoxy[^{3}H]glucose (1μCi/ml) for 15 min at 37ºC. Each experiment was carried out on triplicate wells. Cells were then rapidly rinsed 4 times with ice-cold PBS and solubilized with 1 ml 0.4 N NaOH. 0.5 ml of lysate was transferred into scintillation vials and [^{3}H] measured in a scintillation counter.

Protein expression by Western blotting (III, IV)

Protein expression was quantified in lysates from skeletal muscle cell cultures using the Western blot technique. An aliquot of muscle cell lysate (20 µg protein) was mixed in Laemmli sample buffer containing β-mercaptoethanol. Proteins were separated by 7.5% SDS-PAGE, transferred to polyvinylivenediflouride membrane (Millipore) and blocked in 7.5% non-fat dried milk in Tris-buffered saline with 0.02% tween (TBST) for 2 hours at room temperature. Membranes were incubated overnight at 4°C with antibodies against human ET_A and ET_B receptors (1:200, Alomone Labs), phospho-specific antibodies against phospho-IRS1 Ser^{636} (1:1000), phospho-Akt Ser^{473} (1:1000), phospho-ERK1/2, p42/44 MAPK kinase Thr^{202}/Tyr^{204} (1:1000), phospho-AMPK Thr^{172} (1:1000), Akt (1:1000), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000), or pan-actin (1:1000), all from Cell Signaling Technology). After washing in TBST, the membranes were incubated with horseradish peroxidase anti-rabbit IgG for all the target proteins (1:25000, Bio-Rad) for 1 hour at room temperature, followed by additional washing. Proteins were visualized by enhanced chemiluminescence (Amersham) and quantified using densitometry and Molecular Analyst Software (Bio-Rad).

Study protocols

Study I

All the study subjects participated in two venous occlusion plethysmography protocols (Fig. 3) separated by at least two weeks. In Protocol A, the ET_A receptor antagonist BQ123 (10 nmol/min) was administered. In Protocol B, the combination of BQ123 (10 nmol/min) and the ET_B receptor antagonist BQ788 (5 nmol/min) was given by intra-brachial infusion at a rate of 0.5 ml/min. The order of treatment was randomized. The doses of the antagonists were based on previous studies.[65, 70] On both occasions, basal FBF was recorded during an infusion of saline for two minutes at a rate of 2.5 ml/min. Basal endothelium-dependent and –independent vasodilatation was determined as described above before and following 60 min of ET receptor antagonist infusion. FBF was determined every 10 min during the 60-min infusion of the antagonists. Venous plasma glucose samples were collected before and at 60 min of ET receptor blockade. Blood pressure and heart rate were determined from the arterial catheter at baseline and after the infusions of acetylcholine, SNP and the ET receptor antagonists.

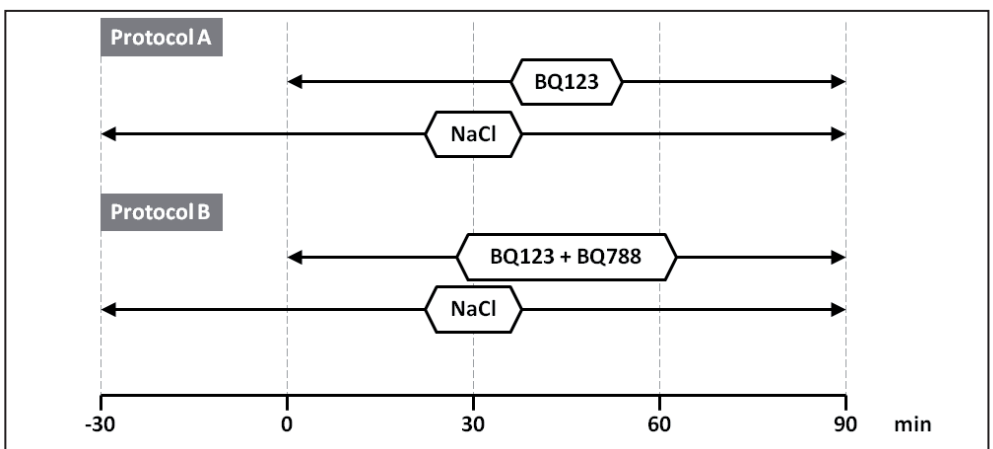

Figure 3. Study protocol for venous occlusion plethysmography in Study I.

Study II

The study consisted of three different hyperinsulinemic-euglycemic clamp protocols: (A) a clamp with an infusion of the ET_A receptor antagonist BQ123, (B) a clamp with a combined infusion of BQ123 and the ET_B receptor antagonist BQ788 and (C) a control clamp with saline infusion. There was at least one week between the clamp studies. The investigations were performed in random order and the patients were unaware of the order of the clamps and were thus blinded to the treatments. In Protocol A, the ET_A receptor antagonist BQ123 was infused at a rate of 5 nmol/kg/min. In Protocol B, the ET_B receptor antagonist BQ788 was infused at a rate of 4 nmol/kg/min, together with BQ123 (5 nmol/kg/min). The infusions of the antagonists started 60 min into the clamp and were maintained for 15 min (Fig. 4). The doses of BQ123 and BQ788 were based on previous studies demonstrating effective hemodynamic responses and antagonism of vascular effects evoked by ET-1.[113, 116-118] In Protocol C, an infusion of saline was started at 60 min into the clamp and was maintained for 15 min. Total body glucose uptake (M) values were calculated during three 20-min periods (Period I, II and III respectively; Fig. 4) during and following the administration of saline/antagonists and then corrected for the mean of the two plasma insulin values obtained during each period (M/I value).

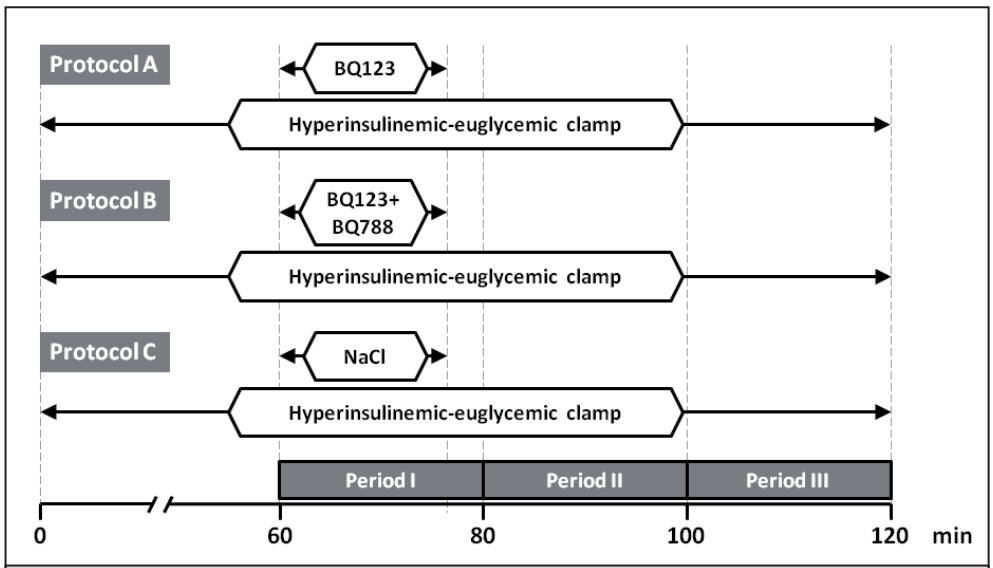

Figure 4. Study protocols for the hyperinsulinemic-euglycemic clamp in Study II. An infusion of saline/ET receptors antagonists was given for 15 min.

Study III

Each patient was investigated on three different occasions using different venous occlusion plethysmography protocols (Fig. 5). The investigations were performed in random order, the subjects were unaware of this order and were thus blinded to the treatments. The investigations were separated by at least one week. In Protocol A, saline was infused for 30 min (60 ml/hour), followed by a 60-min infusion of the ET_A receptor antagonist BQ123

(10 nmol/min) and the ET_B receptor antagonist BQ788 (10 nmol/min). This was followed by a co-infusion of the antagonists and insulin (0.05 mU/kg/min; 20 ml/hour) for another 60 min. In Protocol B, saline was infused for 30 min, followed by an insulin infusion (0.05 mU/kg/min) for 120 min. In Protocol C, saline was infused for 30 min, followed by an infusion of insulin alone for 60 min. This was followed by a co-infusion of ET-1 (20 pmol/min) together with insulin for 60 min. In seven subjects, the ET-1 infusion was prolonged for an additional 15 min to investigate possible time-dependent differences. A saline infusion was given to keep the infusion volume at the same rate (1 ml/min). All infusions were given into the brachial artery. Deep venous and arterial blood samples were collected during the protocols for the determination of forearm glucose uptake.

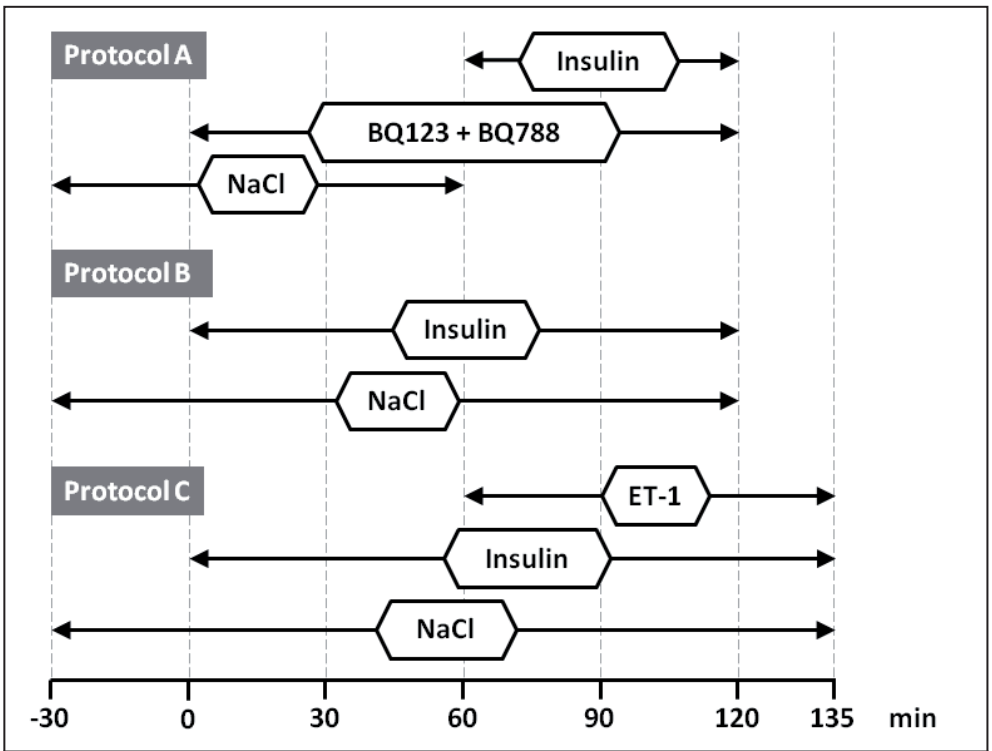

Figure 5. Study protocols for venous occlusion plethysmography in Study III.

Study IV

FBF was assessed using venous occlusion plethysmography. After the insertion of the catheters, an infusion of saline (60 ml/hour) was started, followed by an infusion of ET-1 (20 pmol/min) for 120 min. EDV and EIDV were determined as described above. Arterial and venous blood samples were collected repeatedly during the study protocol for the determination of glucose and insulin levels.

Biochemical analysis

Fasting venous blood samples for total LDL and HDL cholesterol, triglycerides and HbA1c were assessed according to local laboratory routines. In Studies I and II, plasma glucose was analyzed by the azidemethemoglobin method using a HemoCue B-Glucose Analyser (HemoCue, Ängelholm, Sweden) with a precision corresponding to an SD of ± 0.3 mmol/L. In Studies III and IV, plasma glucose was analyzed by a timed endpoint method using Glucose Reagent in conjunction with the SYNCHRON LX® System (Beckman Coulter, Fullerton, USA) with a precision corresponding to an SD of ± 0.11 mM. Plasma insulin was analyzed using an electrochemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany) for the Elecsys® analyzer. CRP was measured using the Behring Nephelometer Analyzer II with a particle-enhanced immunonephelometric assay. ET-1 was analyzed by radioimmunoassay using commercially available antisera (rabbit anti-ET-1 6901, Peninsula, Merseyside, UK) following ethanol extraction.[119] The intra-assay and inter-assay variations were 7% and 10% respectively. The hematocrit was analyzed with a microcapillary hematocrit centrifuge and corrected for trapped plasma.

Statistical analysis

In all the studies, the results are presented as the mean and standard error of the mean (SEM) with the exception for paper I where subject's baseline characteristics data are presented as the mean and standard deviation (SD). Categorical data are expressed as numbers. Two grouping variables were compared by Student's t-test. Mann-Whitney test was used for the comparison of protein expression. Comparison of multiple observations was assessed by one-way ANOVA for repeated measurements with a post-hoc analysis. Differences between the changes in FBF and FGU were assessed by two-way ANOVA. A probability of <0.05 was considered statistically significant. The clinical part of Studies I and II was powered based on an estimate from previous studies.[53, 109, 111] Studies III and IV were powered based on estimates from Studies I and II. Statistical analyses were performed using GraphPad Prism version 4 (GraphPad Software, San Diego, USA).

RESULTS

In vivo studies

ET-1 and vascular function in insulin resistance

Effect of ET receptor blockade on forearm blood flow and endothelial function in insulin-sensitive and insulin-resistant individuals (I, III)

In Study I, basal FBF did not differ significantly between the insulin-sensitive and insulin-resistant groups. The basal (i.e. before the administration of ET receptor antagonists) response to acetylcholine or SNP did not differ between the groups. Selective ET_A receptor blockade did not influence the vasodilator response to acetylcholine or SNP in either group. Dual ET_A/ET_B receptor blockade significantly enhanced EDV in the insulin-resistant group (Fig. 6A) but not in the insulin-sensitive group (Fig. 6B). Furthermore, EDV was significantly greater following dual ET_A/ET_B receptor blockade than following selective ET_A receptor blockade ($p<0.01$). Combined ET_A/ET_B receptor antagonism did not affect EIDV in either the insulin-resistant or insulin-sensitive groups. Selective ET_A receptor blockade slightly yet significantly increased FBF by 18±8% ($p<0.05$) at 60 min of infusion in the insulin-resistant group. Basal FBF was unaffected by dual ET_A/ET_B receptor blockade in either group.

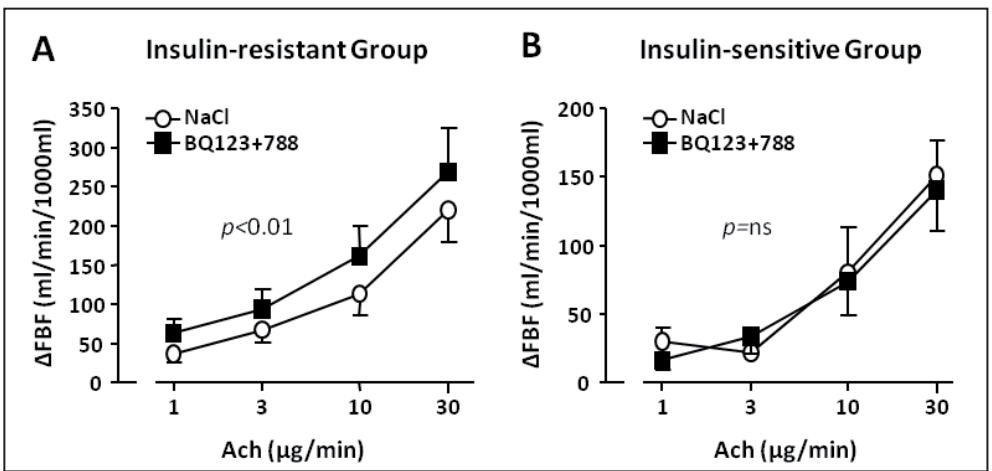

Figure 6. Change in forearm blood flow (FBF) induced by acetylcholine (Ach) during NaCl and following dual ET_A/ET_B receptor blockade by BQ123 and BQ788 in insulin-resistant (**Panel A**; n=11) and insulin-sensitive (**Panel B**; n=8) individuals. A significant difference was found between the responses to Ach during saline and following BQ123 and BQ788 by ANOVA. ns=not significant.

In Study III, there were no significant differences in basal FBF between the three protocols. Dual ET_A/ET_B receptor blockade increased FBF by 30% ($p<0.05$) at 60 min of infusion. The addition of insulin co-infused with the ET receptor antagonists further increased FBF by 16% ($p<0.05$ vs. ET blockade alone). Insulin infusion alone for 120 min did not affect FBF.

Effect of ET-1 on forearm blood flow and endothelial function in insulin-resistant individuals (IV)

The administration of ET-1 reduced FBF by 30% ($p<0.01$) at 60 min and by 36% ($p<0.05$) at 120 min of infusion as compared to basal values. The infusion of ET-1 markedly inhibited acetylcholine-induced vasodilatation ($p<0.001$; Fig. 7A). In addition, the vasodilator response to SNP was slightly yet significantly attenuated by ET-1 (Fig. 7B). Blood pressure and heart rate did not change significantly during the administration of ET-1.

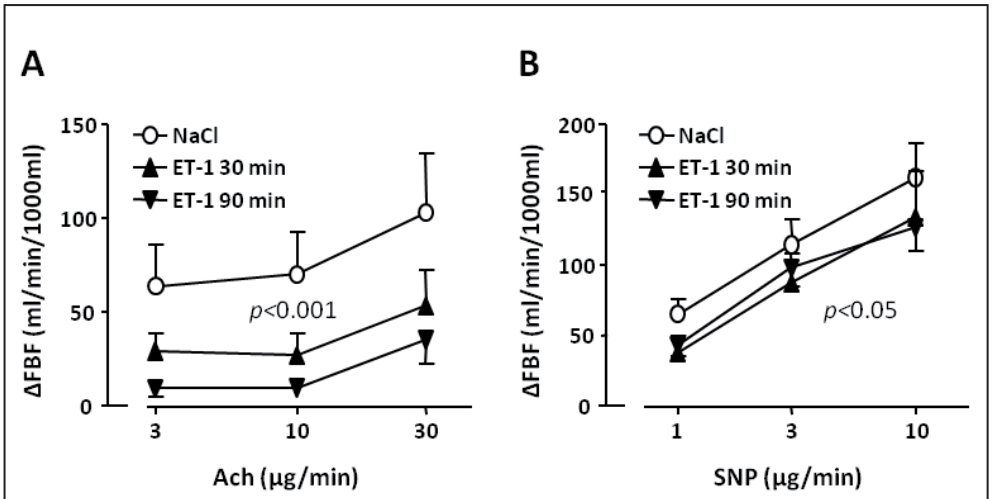

Figure 7. Change in forearm blood flow (FBF) induced by different doses of acetylcholine (Ach; **Panel A**) and sodium nitroprusside (SNP; **Panel B**) during NaCl and following 30 and 90 minutes of ET-1 infusion. Significant differences were found between the responses to Ach and SNP during saline and following ET-1 infusion by ANOVA. Data are means ± SEM (n=9).

Effect of ET receptor blockade on renal and splanchnic blood flow in insulin-resistant individuals (II)

There was no difference in mean arterial pressure (MAP) between the groups at 60 min of hyperinsulinemic-euglycemic clamp (i.e. before the administration of antagonists). However, MAP was reduced following the administration of BQ123 ($p<0.01$) in Protocol A and following the administration of BQ123+BQ788 ($p<0.05$) in Protocol B compared with the saline infusion in Protocol C (control clamp). Renal blood flow (RBF) at baseline did not differ between the protocols and it did not change in the control or BQ123 clamps. In contrast, RBF increased by 24% ($p<0.01$) following the administration of BQ123+BQ788 (Fig. 8A). RBF was significantly higher in Protocol B as compared to the other protocols ($p<0.01$). There were significant differences in renal vascular resistance (RVR) between the clamp protocols (Fig. 8B). RVR was significantly lower in Protocol B than in Protocols A and C ($p<0.05$). There were no significant differences in SBF or splanchnic vascular resistance between the clamp protocols.

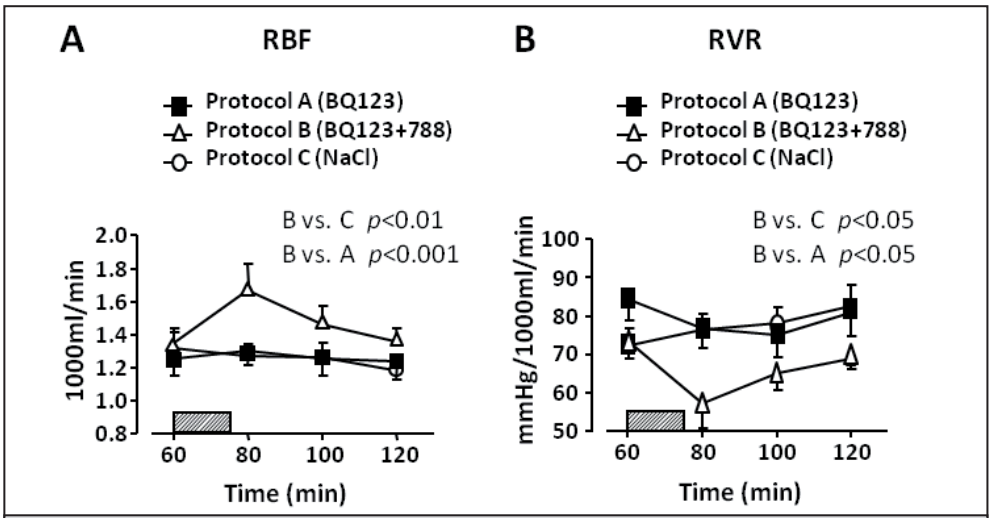

Figure 8. Changes in renal blood flow (RBF; **Panel A**) and renal vascular resistance (RVR; **Panel B**) during selective ET_A receptor blockade (Protocol A), dual ET_A+ET_B receptor blockade (Protocol B) and saline infusion (Protocol C). Hatched bars indicate period of saline/antagonist infusion. Significant differences between groups by ANOVA are indicated. Data are presented as means and SEM; n= 7.

ET-1 and glucose metabolism in insulin resistance

Effect of ET receptor blockade on whole-body glucose uptake and insulin sensitivity in insulin-resistant individuals (II)

Arterial glucose values remained unchanged and did not differ between the clamps. There were no differences in arterial insulin levels between the three clamp protocols. Glucose uptake and insulin sensitivity were determined during saline infusion in Protocol C (control clamp) and following ET receptor blockade in the BQ clamps. There were significant (p<0.05) differences in total body glucose uptake, M values, between the three clamp protocols (Fig. 9A). The M value was significantly higher in Protocol B (BQ123+BQ788 clamp) than in the Protocol C and in comparison with Protocol A (BQ123 clamp; p<0.05). There was no difference in M values between the control and the BQ123 clamps. There were differences in insulin sensitivity, expressed as M/I values, between the three clamps (p<0.02). The M/I value was significantly higher in the BQ123+BQ788 clamp than in the control clamp (p<0.01) and the BQ123 clamp (p<0.05). The M/I value already tended to be higher at the first measurement point during dual receptor blockade with BQ123+BQ788 and it became significantly higher in comparison with the other groups at measurement points 2 and 3 (Fig. 9B). There was no difference between the control clamp and the BQ123 clamp.

Effect of ET-1 and ET receptor blockade on forearm glucose uptake in insulin-resistant individuals (III, IV)

In Study IV, the infusion of NaCl for 30 min did not affect FGU. ET-1 infusion increased the arterio-venous glucose difference by 67% (p<0.05) at 90 min, followed by a decrease to basal values at 120 min (p<0.05 baseline vs. 90 min; Fig. 10A). The infusion of ET-1 decreased FGU

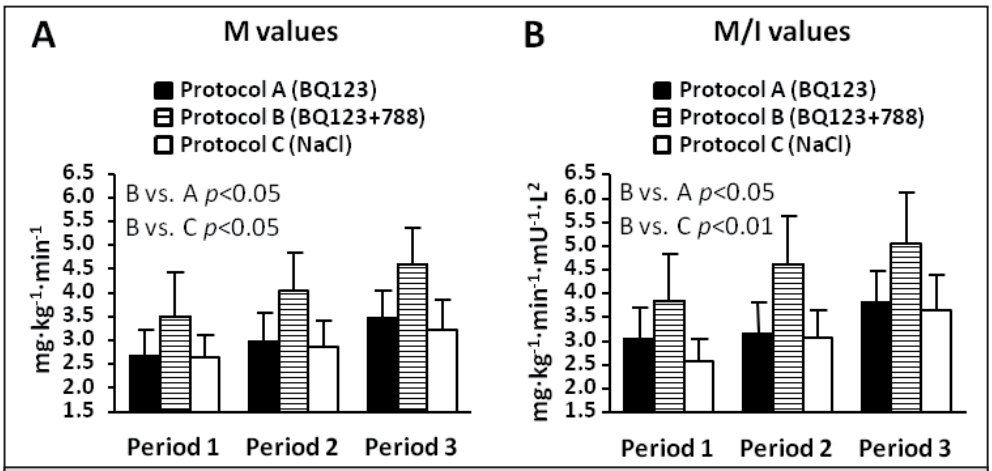

Figure 9. Total body glucose uptake (M; **Panel A**) and insulin sensitivity (M/I; **Panel B**) values in the three sampling periods during selective ET_A receptor blockade in Protocol A (BQ123), during dual ET_A/ET_B receptor blockade in Protocol B (BQ123+788) and during saline infusion in Protocol C (NaCl). Values were calculated during three 20-min periods following the administration of saline/antagonists and were then corrected for the mean of the two plasma insulin values obtained during each period (M/I). Significant differences between groups are indicated. Data are presented as means and SEM; n = 7.

from 5.8±2.0 µmol/min x 1000ml at baseline (saline) to 3.4±0.8 µmol/min x 1000ml at 120 min (p<0.05; Fig. 10B). During the protocol, arterial insulin concentrations decreased from 81.0±11.0 pM at baseline to 65.7±7.5 pM at 120 min of ET-1 infusion (p<0.05). The arterio-venous concentration difference of insulin remained unchanged. There was no correlation between the reductions in arterial insulin concentration and FGU (r=0.05, p=0.91).

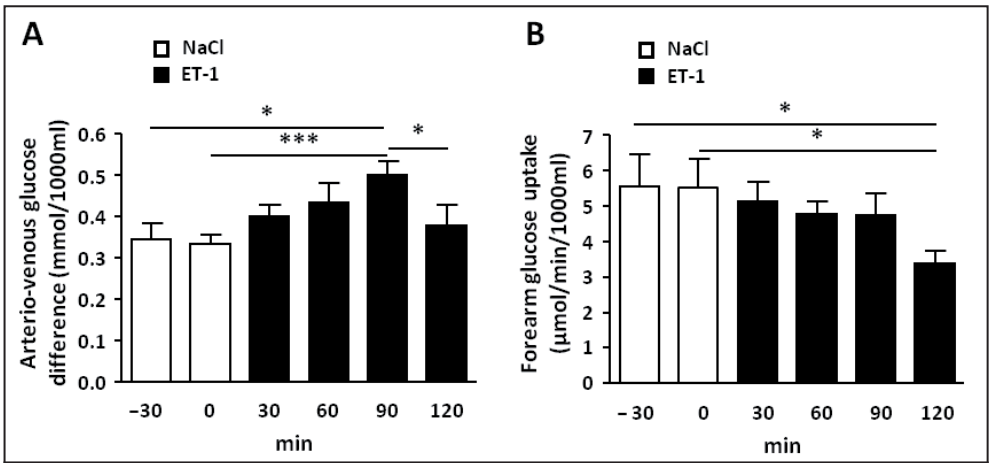

Figure 10. Arterio-venous glucose differences during NaCl infusion and following a 120-min infusion of ET-1 (**Panel A**). Forearm glucose uptake during NaCl infusion and following a 120-min infusion (**Panel B**). *p<0.05, ***p<0.001 by one-way ANOVA. Data are means ± SEM (n=9).

In Study III, the infusion of ET_A/ET_B receptor antagonists in Protocol A increased FGU from 5.1±1.0 at baseline (saline) to 8.3±1.1 µmol/min x 1000ml at 60 min ($p<0.05$; Fig. 11A). The co-administration of insulin further increased glucose uptake to 15.7±1.8 µmol/min/1000ml ($p<0.001$; Fig. 11B). The insulin infusion in Protocol B increased FGU from 4.1±1.1 at baseline (saline) to 7.7±1.9 µmol/min x 1000ml ($p<0.05$) at 30 min and to 12.3±1.6 µmol/min x 1000ml ($p<0.001$; Fig 11B) at 60 min. Dual ET_A/ET_B receptor blockade in combination with insulin (Protocol A) resulted in significantly greater FGU than the infusion of insulin alone (Protocol B) (Fig. 11B). In Protocol C, the infusion of insulin increased FGU from 7.1±0.7 µmol/min x 1000ml at baseline to 17.8±3.0 µmol/min x 1000ml at 60 min of infusion. After 60 min of insulin infusion, glucose uptake reached a steady state. ET-1 infusion for 60 min in combination with insulin did not affect insulin-stimulated FGU (16.5±2.0 µmol/min x 1000ml). Nor did the prolongation of the ET-1 infusion for an additional 15 min in 7 subjects affect FGU.

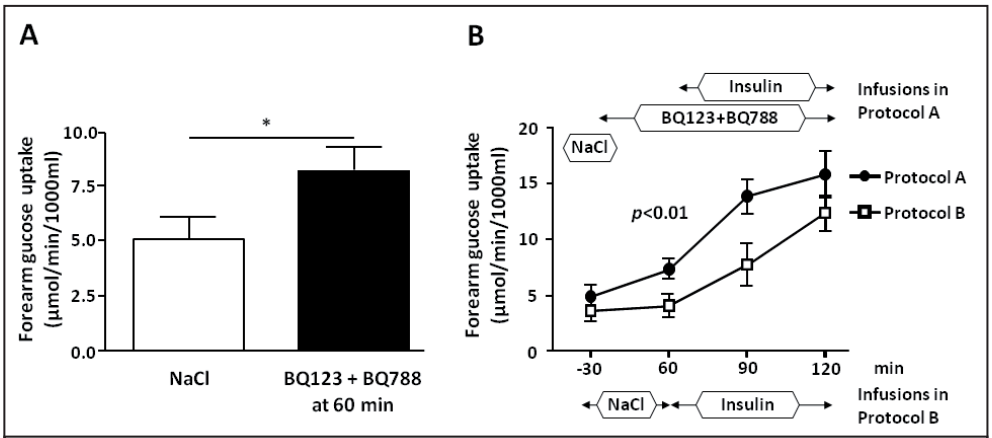

Figure 11. Forearm glucose uptake during NaCl infusion and following a 60-min infusion of the ET_A/ET_B receptor antagonists BQ123 and BQ788 in Protocol A (**Panel A**). *$p<0.05$ using Student's *t*-test. Effect of dual ET_A/ ET_B receptor blockade (BQ) on basal and insulin-stimulated glucose uptake in Protocol A in comparison with the effect of insulin alone in Protocol B (**Panel B**). There was a significant difference between the entire protocols including all time points by two-way ANOVA. Data are means ± SEM (n=10).

In vitro studies

ET receptor expression in skeletal muscle cell cultures (IV)

Western blot analysis revealed the expression of ET_A and ET_B receptors in skeletal muscle cell cultures (Fig. 12A and B). ET_A receptor expression was higher in cells from subjects with type 2 diabetes (T2D) compared with cells from subjects with normal glucose tolerance (NGT) ($p<0.05$; Fig 12A). There was also a trend towards increased expression of ET_B receptors in cells from subjects with T2D ($p=0.13$; Fig 12B).

Effect of ET-1 and ET receptor blockade on glucose uptake in skeletal muscle cell cultures (III, IV)

One hour exposure to ET-1 increased glucose uptake in skeletal muscle cells from NGT subjects (Fig. 13A and B) and this effect persisted at 1, 2 and 6 hours ($p<0.05$; Fig. 13A) of incubation

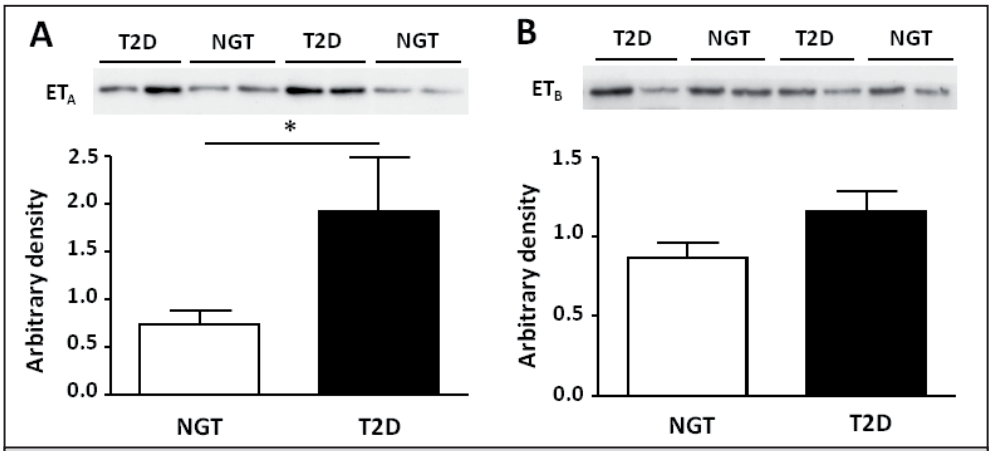

Figure 12. Western blot analysis of the ET_A (**Panel A**) and ET_B (**Panel B**) receptor expression in cells established from subjects with normal glucose tolerance (NGT; n=8) and subjects with type 2 diabetes (T2D; n=8). *p<0.05 using the Mann-Whitney test. Data are means ± SEM.

with ET-1. At 24 hours, ET-1 induced a significant reduction in glucose uptake (Fig. 13A). The co-incubation of ET-1 with bosentan (3 μM) for 24 hours completely prevented the inhibitory effect of ET-1 on glucose uptake. ET-1 did not further increase insulin-stimulated glucose uptake in cells established from subjects with normal glucose tolerance (Fig. 13B). In contrast, ET-1 or insulin did not increase glucose uptake in cells from T2D subjects (Fig. 13B).

Effect of ET-1 on insulin signaling

Incubation with ET-1 for 1 hour reduced insulin-stimulated Akt phosphorylation in a dose-dependent manner (Fig. 14A). This inhibitory effect of ET-1 was reversed following the

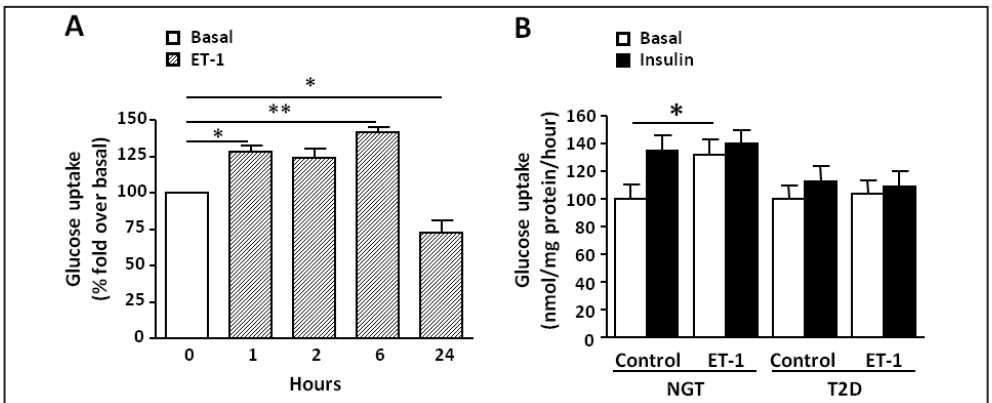

Figure 13. Panel A. Glucose uptake in skeletal muscle cells at basal state and after 1, 2, 6 and 24 hours treatment with 10 nM ET-1. *p<0.05 using one-way ANOVA. Data are means ± SEM (n=3). **Panel B.** Basal and insulin-stimulated glucose uptake in cells established from subjects with normal glucose tolerance (NGT; n=11) and subjects with type 2 diabetes (T2D; n=11) under basal conditions and after 1 hour of ET-1 treatment. *p<0.05 using Student's t-test. Data are means ± SEM.

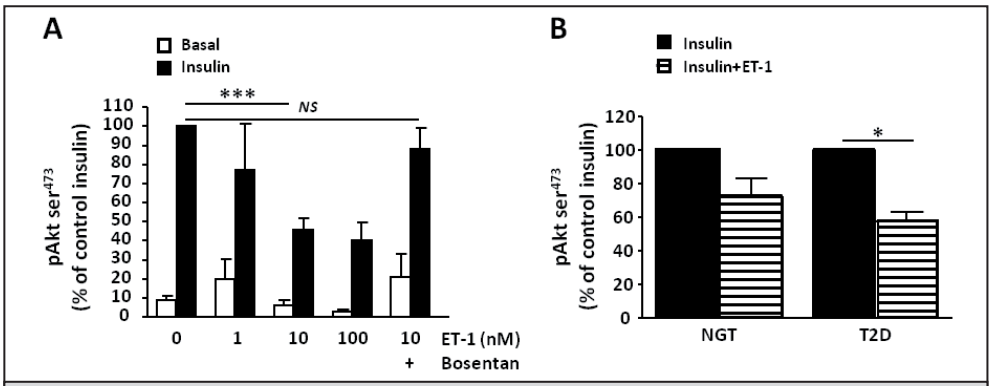

Figure 14. Panel A. The basal and insulin-stimulated expression of phosphorylated Akt under control conditions and after 1 hour exposure to ET-1 (1, 10 and 100 nM) in the absence and presence of the ET receptor antagonist bosentan (3 μM). ***p<0.001 using a one-sample t-test. *NS*=not significant. Data are means ± SEM (n=4-19). **Panel B.** The insulin-stimulated expression of phosphorylated Akt under control conditions and after 1 hour exposure to ET-1 (10 nM) in cells established from subjects with normal glucose tolerance (NGT; n=5) and subjects with type 2 diabetes (T2D; n=4). *p<0.05 using a one-sample t-test. Data are means ± SEM.

pre-exposure of muscle cultures to the dual ET_A/ET_B receptor antagonist bosentan (Fig. 14A). The effect of ET-1 on insulin-stimulated Akt phosphorylation was evident in cells established from both NGT and T2D subjects (Fig. 14B). ET-1 did not affect the basal or insulin-stimulated phosphorylation of Akt following 24 hours incubation.

One hour and 24 hours exposure to ET-1 increased the phosphorylation of IRS1 Ser^{636}, an effect that was blocked by the pre-exposure of cells to bosentan (Fig. 15A and B). ET-1 exposure transiently increased the phosphorylation of ERK1/2, peaking at 5 min and returning to baseline by 20 min. No effect of ET-1 was noted on the phosphorylation of AMPK at time points from 3 to 20 min up to 24 hours (data not shown).

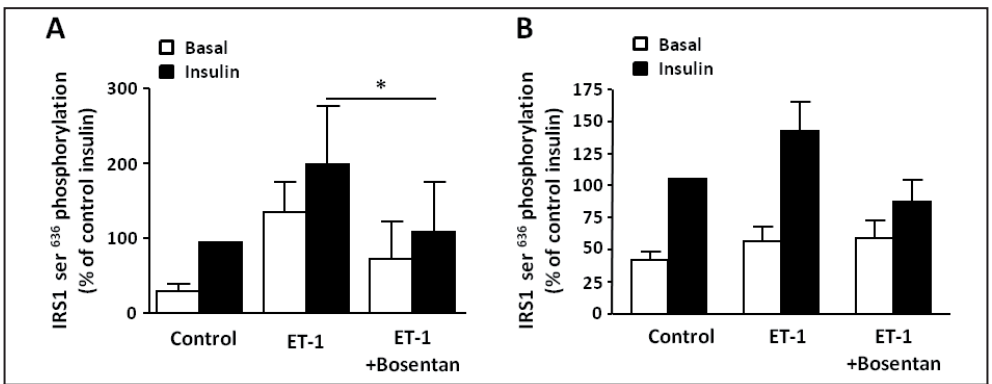

Figure 15. Panel A. The basal and insulin-stimulated expression of phosphorylated IRS1, under control conditions and after 1 hour exposure to ET-1 in the absence and presence of the ET receptor antagonist bosentan (3 μM). *p<0.05 using the Mann-Whitney test. Data are means ± SEM (n=5). **Panel B.** Basal and insulin-stimulated IRS-1 ser^{636} phosphorylation under control conditions and after 24 hours exposure to ET-1 (10 nM) or a combination of ET-1 and bosentan (3 μM). Data are means ± SEM (n=3).

General Discussion

Effects of ET-1 on blood flow and vascular function in insulin-sensitive and insulin-resistant individuals

Growing evidence suggests that ET-1 plays an important role in the development of endothelial dysfunction. The major mechanism for this pathophysiological process is the reduction of NO bioavailability: ET-1 either reduces NO production (via the inhibition of eNOS activity) or increases its degradation (via the formation of oxygen radicals). It has been suggested that the increased production of ET-1 is part of the endothelial dysfunction in insulin resistance.[33] In fact, there are a number of studies demonstrating that different pathological conditions associated with the presence of insulin resistance, such as type 2 diabetes,[48] obesity,[49] essential hypertension[50] and coronary artery disease,[51] are associated with elevated plasma levels of ET-1. Despite the fact that elevated circulating plasma levels of ET-1 were not found in insulin-resistant individuals in Study I, dual ET_A/ET_B receptor blockade enhanced the vasodilator response to acetylcholine in this group. Since the vasodilator response to acetylcholine is dependent on the release of endothelium-derived NO,[53] the present observation suggests that dual ET_A/ET_B receptor blockade enhances NO availability in subjects with insulin resistance. Endothelium-dependent vasodilatation was unaffected by either selective ET_A or dual ET_A/ET_B receptor blockade in insulin-sensitive individuals matched for age and gender. Taken together, these data could indicate the presence of enhanced activity of endogenous ET-1 in insulin-resistant subjects without clinical signs of cardiovascular disease. Moreover, the insulin-resistant subjects included in this study did not display impairment in basal endothelium-dependent vasodilatation, indicating that increased ET-1 activity is an early vascular finding in insulin resistance. In Study IV, we demonstrate that exogenous ET-1 impairs both endothelium-dependent and -independent vasodilatation in the forearm of subjects with insulin resistance. This result extends previous findings that exogenous ET-1 markedly reduces endothelium-dependent vasodilatation in healthy subjects.[53]

In Study II, we observed that dual ET_A/ET_B but not selective ET_A, receptor blockade increased renal blood flow and reduced renal vascular resistance in subjects with insulin resistance and cardiovascular disease. These changes in hemodynamic parameters support a role for ET-1 in the regulation of vascular tone in this patient group. Splanchnic blood flow and splanchnic vascular resistance were unaffected by ET antagonists, suggesting regional differences in the influence of ET-1 on vascular tone. However, these results contrast with those previously obtained in patients with chronic renal failure and hypertension, where selective ET_A, but not dual ET_A/ET_B, receptor blockade increased renal blood flow and reduced renal vascular resistance.[117] Important differences between these studies are that patients in our study had normal renal function, based on serum creatinine levels, and normal blood pressure. In Study III, dual ET_A/ET_B receptor blockade significantly increased forearm blood flow in insulin-resistant individuals. Interestingly, the co-infusion of ET receptor antagonists with insulin resulted in a significant additional increase in blood flow, despite the fact that insulin did not affect forearm blood flow, which is in accordance with previous studies.[120, 121] Although a carry-over effect by the ET receptor blockade cannot be ruled out, this observation may indicate the unmasking of insulin-mediated vasodilatation. These data are in agreement with previous experimental studies, suggesting that insulin induces vasodilatation only after the

blockade of ET receptors in insulin resistance,[122, 123] via an effect coupled to an increase in NO bioavailability.[124]

It is often assumed that most of the pathological effects of ET-1 are mediated via the stimulation of the ET_A receptor and that the blockade of endothelial ET_B receptors may have a detrimental effect on vascular function.[57] Despite this, in Study I, selective ET_A receptor blockade did not influence endothelium-dependent vasodilatation in the insulin-resistant group. It could therefore be speculated that the blockade of both the ET_A and the ET_B receptor is needed to enhance endothelial function in this group. The present study extends the findings of improved endothelium-dependent vasodilatation by dual ET_A/ET_B receptor blockade in patients with hypertension[55] and atherosclerosis.[54] It could be hypothesized that this effect is related to the increased expression of ET_B receptors, which has been reported in atherosclerotic human arteries.[67] The finding that dual ET_A/ET_B receptor blockade did not result in impaired vascular function in the healthy insulin-sensitive group, despite the blockade of the ET_B receptor, is also of importance. The exact mechanism behind the effect of ET receptor blockade on endothelium-dependent vasodilatation is not fully understood and could be attributed to several factors. One possibility is that ET receptor blockade up-regulates NOS. The dual ET receptor antagonist bosentan was shown to increase the expression of endothelial NOS.[56] Furthermore, NOS activity is stimulated by dual ET receptor blockade.[57] Another possibility is that ET-1 increases the formation of reactive oxygen species which will result in the reduced bioactivity of NO.[125-127] ET receptor antagonism may therefore enhance endothelium-dependent vasodilatation via the reduced formation of reactive oxygen species. From Studies I, II, III and IV, it could be concluded that ET-1 impairs basal vascular tone and endothelium-dependent vasodilatation in subjects with insulin resistance. Dual ET_A/ET_B but not selective ET_A, receptor blockade improves endothelial function and increases basal blood flow in this group.

ET-1 and glucose metabolism in insulin resistance *in vivo* and *in vitro*

Several studies indicate that ET-1 is able to interfere with glucose metabolism. The administration of exogenous ET-1 or its precursor, big ET, was shown to reduce insulin sensitivity in healthy humans[110, 111] and in experimental animals.[108] Furthermore, a negative correlation between total glucose uptake and circulating ET-1 levels was found in diabetic patients.[48] It has been reported that exogenous ET-1 exerts a biphasic effect on glucose metabolism in the perfused rat hind limb with the stimulation of glucose uptake at a low dose and the inhibition of glucose uptake at a high dose.[128] ET_A receptor blockade was shown to increase glucose uptake during a hyperinsulinemic-euglycemic clamp in obese individuals.[112] Studies of isolated skeletal muscle strips suggest that this effect is independent of a reduction in skeletal muscle blood flow and implies a direct influence by ET-1 on insulin-stimulated glucose transport.[108] Accordingly, ET-1 reduces the expression of IRS1 and insulin-stimulated Akt phosphorylation in skeletal muscle vascular smooth muscle cells.[107, 108] Furthermore, ET-1 impairs glucose transporter GLUT4 trafficking in rat adipocytes.[106]

Based on these previous observations, we hypothesized that endogenous ET-1 affects insulin-stimulated glucose uptake in patients with insulin resistance and coronary artery disease. In Study II, we demonstrated that dual ET_A/ET_B receptor blockade acutely increased total body glucose uptake and insulin sensitivity in obese patients with insulin resistance and coronary artery disease. On the other hand, no improvement was observed following selective ET_A receptor blockade. This is in accordance with the finding in Study I, where dual ET_A/ET_B

receptor blockade, but not selective ET_A, improved endothelium-dependent vasodilatation. These observations indicate that ET-1 contributes to insulin resistance and that both the ET_A and the ET_B receptor need to be blocked to achieve this effect.

Since skeletal muscle is a primary site of glucose uptake, we used the forearm model in which it is possible to determine local arterio-venous concentration differences and blood flow to calculate glucose uptake in a vascular bed mainly supplying skeletal muscle[114] in Studies III and IV. In Study III, dual ET_A/ET_B receptor blockade not only increased insulin-stimulated skeletal muscle glucose uptake in subjects with insulin resistance, it also significantly increased glucose uptake *per se*. When the ET receptor antagonists were co-infused with insulin, glucose uptake was significantly greater than during the infusion of insulin alone. Collectively, these observations suggest that dual ET_A/ET_B receptor blockade augments basal glucose uptake and facilitates insulin-stimulated glucose uptake in the skeletal muscle tissue of subjects with insulin resistance. In Study III, the administration of endogenous ET-1 for 60 min did not affect forearm glucose uptake, which is in contrast to previous observations showing that the administration of exogenous ET-1 or its precursor, big ET, reduces insulin sensitivity in healthy humans[110, 111] and in experimental animals.[108] We therefore hypothesized that the lack of ET-1 effect on forearm glucose uptake could be attributed to the constant intra-arterial infusion of insulin, which is known to increase blood flow in an NO-dependent manner and to stimulate glucose uptake in skeletal muscle.[83] To elucidate this, we designed Study IV, where we used the same forearm model to study the independent metabolic actions of ET-1 in the same subjects. In this study, the exogenous infusion of ET-1 resulted in a significant reduction in forearm glucose uptake following 2 hours infusion. Despite an overall reduction in forearm glucose uptake, arterio-venous glucose concentrations were significantly increased during the first 90 min of ET-1 infusion, after which they fell to basal levels at 2 hours. This could indicate an initial ET-induced stimulation of glucose uptake in skeletal muscle, which may be obscured by the parallel reduction in blood flow. One limitation of this study is that we did not obtain steady-state insulin levels. During the protocol, arterial insulin levels fell by 19%. The underlying cause of this change is unknown, but it may partly explain the reduction in glucose uptake. However, no correlation between the reduction in insulin levels and the ≈ 40% reduction in forearm glucose uptake was observed. The reduction in forearm glucose uptake may therefore be related to other factors such as direct effects by ET-1 on skeletal muscle insulin signaling and blood flow.

To obtain further insight into the complex nature of these changes *in vivo*, we studied the effect of ET-1 in cultured human skeletal muscle, giving us an opportunity to determine any direct and flow-independent effects of ET-1 on glucose uptake. In Study IV, we demonstrated that both ET_A and ET_B receptors are expressed on skeletal muscle cells and that there is therefore a rationale for a direct effect of ET-1 in cell cultures. In Study III, 24 hours incubation with ET-1 induced a marked inhibition of basal and insulin-stimulated glucose uptake in cultured skeletal muscle cells, obtained from healthy subjects. This observation is in accordance with previous data in adipocytes[129] and indicates that ET-1 can exert its metabolic properties independent of its vasoconstricting effects. The inhibitory effect of ET-1 on glucose uptake was completely antagonized by dual ET_A/ET_B receptor blockade, suggesting a receptor-mediated effect of ET-1, and this supports our *in vivo* observation in Study III. In Study IV, we investigated the temporal effect of ET-1 on glucose uptake in skeletal muscle cell cultures. In cells obtained

from healthy, normal glucose-tolerant subjects, acute ET-1 exposure (1 hour up to 6 hours exposure) increased glucose uptake. This observation is in agreement with previous data obtained in rodent muscle.[108] It is therefore possible to speculate that, in skeletal muscle, ET-1 exerts a biphasic effect with an initial stimulation, followed by a reduction in glucose uptake. Interestingly, in Study IV, incubation with ET-1 did not affect glucose uptake in cells cultured from individuals with type 2 diabetes, indicating a quantitative and qualitative difference in the effect of ET-1 on glucose metabolism between insulin-sensitive and insulin-resistant states. Furthermore, the expression of ET_A receptors was increased in cells from subjects with type 2 diabetes. Whether this difference influences alterations in glucose uptake remains to be elucidated in further studies. Collectively, the data indicate that prolonged exposure to ET-1 reduces total body and skeletal muscle glucose uptake and that this effect is antagonized by dual ET_A/ET_B receptor blockade.

ET-1 and insulin signaling

We also aimed to dissect molecular signaling events underlying the ET-1 effects on glucose uptake. Since the metabolic IRS1-PI3 kinase-Akt branch of insulin signaling pathways plays a pivotal role in promoting insulin-mediated glucose uptake,[95] it is tempting to speculate that an ET-1-mediated reduction in skeletal muscle glucose uptake occurs via the modification of IRS1 and Akt phosphorylation. In Studies III and IV, both acute and prolonged ET-1 exposure increased IRS1 serine phosphorylation on a negative regulating site, Ser^{636}, which acts as a negative feedback control mechanism reducing IRS1 tyrosine phosphorylation following prolonged insulin exposure.[130] This effect was prevented by the ET_A/ET_B receptor antagonist bosentan. In Study IV, we demonstrate that cells incubated with ET-1 for 1 hour reduced insulin-stimulated Akt phosphorylation via a dose-dependent and receptor-dependent mechanism. This observation is in line with previous observations in rat skeletal muscle and vascular smooth muscle cells.[107, 108] However, this is not the case in adipocytes, in which ET-1 mediates an impairment in glucose transport via PI3K-independent mechanisms.[106, 131] In Study III, we observe that this inhibitory effect of ET-1 on Akt phosphorylation is diminished after the prolonged 24 hours incubation. Furthermore, in Studies III and IV, a reduction in glucose uptake in skeletal muscle cells derived from healthy subjects was only evident following 24 hours of ET-1 exposure.

Akt has been shown to play an important role in promoting the metabolic actions of insulin, including GLUT4 translocation.[132] Despite this, there is increasing evidence that Akt activation alone is not sufficient for insulin effects on glucose transport.[133-135] Furthermore, the insulin-mediated stimulation of Akt is not impaired in the skeletal muscle of insulin-resistant subjects.[136] The relationship between insulin action and Akt activity, and subsequent downstream events, therefore appears to be complex and is incompletely understood. It is likely that the deregulation of additional pathways is required for ET-induced impairment in skeletal muscle glucose uptake. We hypothesized that the acute ET-1-mediated increase in glucose uptake observed in cultured skeletal muscle was due to a transient stress response, since ET-1 alone did not increase Akt phosphorylation and is thus unlikely to utilize an insulin-like signaling cascade. However, the exposure of skeletal muscle cells to ET-1 did not increase the phosphorylation of AMPK and the molecular signal mediating the increased glucose uptake remains to be determined. The acute exposure of skeletal muscle cells to ET-1 induced a transient increase in the phosphorylation of ERK MAP kinase, which is associated with cell proliferation and

migration, as well as the excessive formation of reactive oxygen species.[137] ET-1 exposure did not alter GLUT1 and GLUT4 gene expression in cultured human muscle cells. Our data from Studies III and IV therefore suggest that ET-1-mediated effects on glucose transport in cultured human muscle cells may be dependent on signaling downstream of IRS1 Ser^{636} but not dependent on Akt, ERK or AMPK.

CONCLUSIONS

1. ET-1 impairs basal vascular tone and endothelium-dependent vasodilatation in subjects with insulin resistance. Dual ET_A/ET_B but not selective ET_A receptor blockade improves endothelial function and increases basal blood flow in this group.

2. ET-1 is involved in the regulation of total body and skeletal muscle glucose uptake in subjects with insulin resistance and this negative effect is antagonized by dual ET_A/ET_B, but not selective ET_A, receptor blockade.

3. Prolonged exposure to ET-1 reduces glucose uptake in insulin resistant subjects as well as in cultured human skeletal muscle cells. Dual ET_A/ET_B receptor antagonism improves skeletal muscle glucose uptake.

4. ET-1 reduces glucose uptake via a receptor-dependent pathway which seems to be dependent on signalling downstream of IRS1.

Collectively, the obtained data suggest that ET-1 is of pathophysiological importance for the development of endothelial dysfunction and contributes to glucometabolic perturbations in subjects with insulin resistance. Dual ET_A/ET_B receptor blockade may be a potential therapeutic target in order to improve endothelial function and insulin sensitivity in this patient group.

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